BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

ECCA

Practical Immunology

LESLIE HUDSON
Department of Experimental Immunobiology
Wellcome Research Laboratories
Beckenham, Kent, England
Formerly at the
Basel Institute for Immunology
Switzerland

FRANK C. HAY
Department of Immunology
Middlesex Hospital Medical School
London, England

FOREWORD BY
SIR MACFARLANE BURNET

BLACKWELL SCIENTIFIC PUBLICATIONS
OXFORD LONDON EDINBURGH MELBOURNE

[redacted]

Exhibit 1

Osney Mead, Oxford,
8 John Street, London WC 1
9 Forrest Road, Edinburgh,
P.O. Box 9, North Balwyn, Victoria, Australia.

ISBN 0 632 00211 5

First published 1976

Distributed in the United States of America by
J.B. Lippincott Company, Philadelphia
and in Canada by
J.B. Lippincott Company of Canada Ltd, Toronto.

Printed in Great Britain
at the Alden Press, Oxford
and bound by
Kemp Hall Bindery, Oxford

8 Pure antibodies—Pure cells

8.1 PURE ANTIBODIES

As we know from Chapter 5 an antibody reacts specifically with its own *antigenic determinant* to form an antigen–antibody complex.

If an animal is immunized with an antigen it will respond with antibodies all reacting with the antigen to some degree. Serum from this animal will have the usual range of immunoglobulins but those reacting with this antigen will be at a relatively higher concentration, compared to normal serum.

To study a particular antibody in detail it is of great advantage to be able to separate it from the surrounding, non-specific antibody molecules. The precipitated antigen–antibody complex has already done this for us! Unfortunately, the antibody in combination with its antigen has already completed the interesting reactions before we could follow them.

We must separate the complex, remove the antigen and we will then have purified antibody in a reactive state.

The forces binding antibody to antigen are those involved in any protein–protein interaction:

1 Coulombic
2 Dipole
3 Hydrogen bonding
4 van der Waals
5 Hydrophobic bonding

All these forces depend on the charge of the molecules taking part in the reaction; the net charge of the molecules in turn depends on the pH of the medium. If the pH of the medium is lowered sufficiently the protein molecules change conformation, gain H^+ ions and so repel each other. We are now faced with the problem of physically removing the antigen or the antibody, because when the pH is returned to neutrality the complexes would re-form.

If the antigen is *insoluble* it can be removed easily.

There are many methods available for rendering either the antigen or antibody insoluble, some of which are described in the following sections. The antigen in its insoluble form is known as the IMMUNOADSORBENT and the whole purification process is AFFINITY CHROMATOGRAPHY.

8.1.1 PREPARATION OF IMMUNOADSORBENT

In this experiment antibodies to mouse immunoglobulin are purified but the identical method can be used for other proteins.

Materials and equipment

Sepharose 4B (Appendix II)
Cyanogen bromide (THIS CHEMICAL IS VERY DANGEROUS AND **MUST** BE HANDLED IN A FUME CUPBOARD)
2·0 N sodium hydroxide
Phosphate buffered saline (PBS) (Appendix I)
Borate saline buffer, pH 8·3, ionic strength 0·1 (Appendix I)
Mouse immunoglobulin (Section 1.2.2c)

Method

1 Pipette 14 ml of Sepharose (about 200 mg) into a 50 ml glass beaker and add 10 ml of distilled water.

ALL PROCEDURES MUST NOW BE CARRIED OUT IN A FUME CUPBOARD

2 Weigh a stoppered tube, add some solid cyanogen bromide, replace the stopper and re-weigh the tube.
3 Dissolve the cyanogen bromide in distilled water to a final concentration of 50 mg ml^{-1}.
4 Place the Sepharose beads on a magnetic stirrer and raise the pH to 11·0–11·5 with 2·0 N NaOH.
5 Add 10 ml of the cyanogen bromide solution (USE A PIPETTE SAFETY BULB).
6 Maintain the pH at 11·0–11·5 by dropwise addition of sodium hydroxide for 5–10 min until the pH becomes stable.
7 Wash the activated beads on a sintered glass funnel with 100 ml of water, and then 100 ml of borate buffered saline.
8 Wash the beads into a glass beaker, allow them to settle and remove the supernatant.
9 Add 100 mg of mouse immunoglobulin at 5–10 mg ml^{-1} (initial concentration).
10 Leave the beads stirring with the protein overnight at 4° (most of the uptake occurs within the first 4 hours and so this stage can be abbreviated).
11 Wash the beads on a sintered glass funnel with 10 ml PBS and collect the washings. (Use negative pressure and collect washings in a tube standing in a side arm flask.)
12 Wash the beads thoroughly with PBS to remove the rest of the unbound immunoglobulin.

13 A UV spectrophotometer reading of the washings will give the amount of unbound protein and so the approximate quantity of protein bound to the column can be calculated.

The immunoadsorbent is now ready. Store in PBS containing azide (0·1 M)

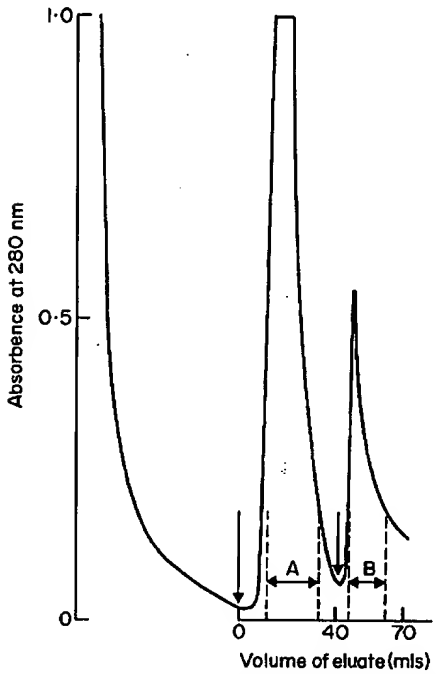


Fig. 8.1. Elution of antibody from an immunoadsorbent with glycine-HCl buffer
The unbound protein was washed away with phosphate buffered saline until the absorbance at 280 nm was below 1·0%. A first population of antibody molecules was eluted with 0·1 M, pH 2·5 glycine–HCl buffer (first arrow). When elution was complete a second population of antibody molecules was eluted off with the same buffer containing 10% dioxane (this second elution was started at the second arrow). To reduce the total sample volume only the volumes A and B were collected.

8.1.2 USE OF IMMUNOADSORBENT FOR ANTIBODY PURIFICATION

Materials and equipment

Rabbit anti-mouse immunoglobulin (Section 1.4.2)
Immunoadsorbent—mouse Ig on Sepharose 4B (Section 8.1.1)
Glycine-HCl buffer, 0·1 M, pH 2·5 (Appendix I)
Trichloracetic acid, 10% aqueous solution (TCA)
Tris (hydroxymethyl) aminomethane
Phosphate buffered saline (PBS) (Appendix I)
Chromatography column (Appendix II)

of the washings will give the
he approximate quantity of
lculated.
y. Store in PBS containing

0
ıls)
lsorbent with glycine–HCl buffer
)hosphate buffered saline until the
population of antibody molecules
fer (first arrow). When elution was
ecules was eluted off with the same
elution was started at the second
only the volumes A and B were

T FOR ANTIBODY

ction 1.4.2)
rose 4B (Section 8.1.1)
)endix I)
on (TCA)

ıdix I)

Method

1 Pour the immunoadsorbent into the column and equilibrate with 20 ml PBS. Close the column.
2 Run 20 ml of antiserum through the column—do not use positive pressure—allow it to run under 1*g*.
3 Wash the unbound protein from the column. If a Uvicord or similar flow-through UV cell is available, wash the column until the % absorbance is $<1{\cdot}0$ (see Fig. 8.1), otherwise wash with 200 ml PBS. Close the column.

We now have the antigen–antibody complex.

Dissociation of complex

1 Pipette out twenty 0·5 ml aliquots of TCA into small glass tubes. (Use this to sample the effluent for protein during elution if a flow-through UV cell is not available.)
2 Add glycine-HCl buffer to the top of the column and collect the effluent when protein is first detected.
3 Stop collecting the effluent when protein is no longer detectable.

The first stage of the elution is now complete and part of the antibody has been recovered. The low pH will, however, eventually denature the antibody so we must raise the pH.

4 Titrate the protein solution to pH 8·5 with solid Tris. Mix thoroughly and monitor with a pH meter or indicator papers.

We are now going to alter the elution conditions to recover a second batch of antibody.

5 Add glycine-HCl + 10% dioxane to the column. Monitor the effluent and collect the second batch of antibody.
6 Adjust the pH to 8·5 with solid Tris.
7 Read the absorbance of each protein solution at 280 nm and calculate the recovered protein. (Remember to use the correct reference solution for the spectrophotometer!)
8 Concentrate the samples in dialysis tubing against either sucrose or polyethylene glycol 40,000 or by negative pressure dialysis (Fig. 8.2).
9 When the sample volume has been reduced to 3–5 ml, dialyse against 5 l of PBS overnight.
10 Spin off the precipitate and determine the protein content of each sample.

This method of antibody purification is highly reproducible and so it is not necessary to calculate the antibody content of the sample routinely. However, a specimen calculation is given below.

8.1.3 CALCULATION OF RECOVERY FROM IMMUNOADSORBENT

Total weight of immunoglobulin on column = 92·0 mg on 200 mg of Sepharose 4B.

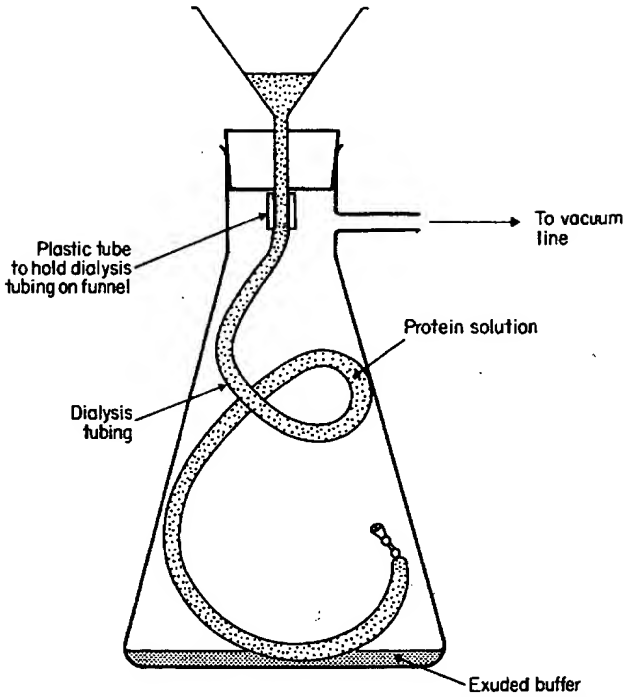


Fig. 8.2. Equipment for the rapid concentration of protein solutions by negative pressure dialysis
It is advisable to test the system for leaks using phosphate buffered saline before adding the protein solution to the dialysis tubing.

Eluates from immunoadsorbent

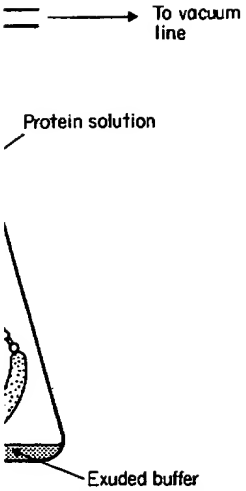
Eluant	Total protein concentration in eluate: immediately		Total protein concentration in eluate: after concentration and dialysis	
Glycine-HCl	36·4 mg	42·4 mg	23·0 mg	25·0 mg
Glycine-HCl + 10% dioxane	6·0 mg		2·0 mg	

Volume of antiserum for antibody purification = 10 ml.

Antibody content of serum calculated from Fig. 8.3, at equivalence conditions as in Section 5.3.1:

Antibody content of serum = 5·2 mg ml^{-1}.

% yield of antibody from serum:
immediately 81·5%
after concentration and dialysis 47·8%



tion of protein solutions by negative

ing phosphate buffered saline before ıbing.

rotein concentration in eluate

	after concentration and dialysis	
ately		
42·4 mg	23·0 mg 2·0 mg	25·0 mg

purification = 10 ml.
ated from Fig. 8.3, at equiva-

; ml^{-1}.

%

Calculation of antibody content of eluate

From Fig. 8.3: Weight of antibody in 200 μg of eluted protein
= 490 − 160
= 230 μg.

Hence all the recovered protein has retained antibody activity. (In general, at least 90% of the recovered protein should be antibody.)

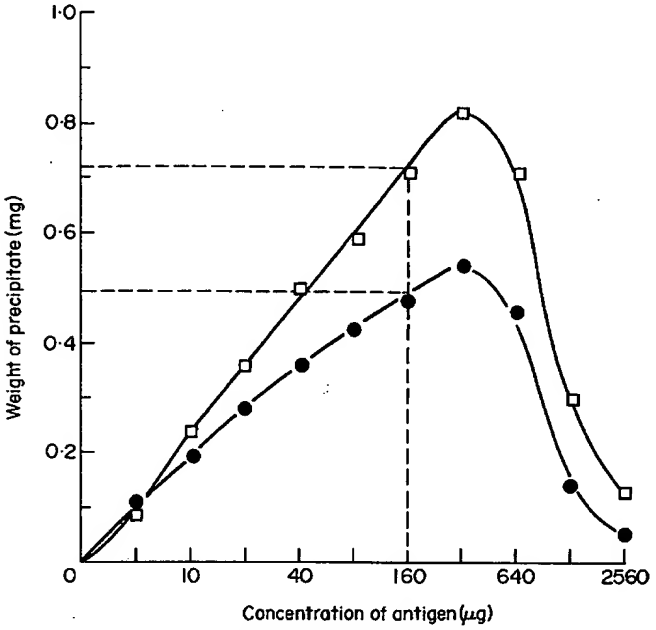


Fig. 8.3. Precipitin curves of anti-immunoglobulin antiserum and antibody
□——□ 0·1 ml of original serum.
●——● 200 μg of purified antibody.

8.1.4 EXPERIMENTS ON PURIFIED ANTIBODY

1 Calculate the yield and antibody content of the sample as in the example using precipitin curves (Section 5.3.1).
2 The antibody eluted with dioxane and buffer is said to be of higher affinity than that eluted by buffer alone (see Fig. 8.2). Investigate this by the method detailed in Section 5.2.3, using goat anti-rabbit immunoglobulin to precipitate the antigen–antibody complex.
3 The purified antibody will be used to isolate B cells in Section 8.2.1.

Technical notes

1 Under the conditions described, the Sepharose should bind 90–100 mg of mouse immunoglobulin. Approximately the same uptake

can be expected with other common antigens, with the notable exception of bovine serum albumin where only 20–30 mg are bound.

2 Although the proportion of antibody in the final sample is fairly constant, the actual yield of antibody relative to the serum concentration varies with serum pool and species. The greatest loss of antibody occurs due to denaturation and precipitation after elution, concentration and dialysis.

The yield of purified antibody from sheep antisera is extremely poor by the elution system described. Recently, 0·1 M citrate buffer, pH 3·0 (Appendix I) has been used with reasonable recovery rates. It is possible that denaturation and precipitation even with rabbit antisera may be reduced using this buffer for elution.

3 In this experiment the immunoadsorbent has been used below its maximal capacity; in general it should be able to deplete 1 ml of antiserum for each mg of antigen on the column.

4 Pre-activated Sepharose is available commercially (Appendix II) this avoids the use of cyanogen bromide. For large-scale preparations, however, it is relatively expensive.

8.1.5 PRACTICAL APPLICATIONS OF IMMUNOADSORBENTS

Besides their use for isolation of pure antibodies, immunoadsorbents are widely used to render antisera specific by depletion of cross-reacting antibodies (Section 1.6.6), and for quantitative adsorption (Section 9.1).

Although the method described used an antigen immunoadsorbent to isolate antibody, it is possible to prepare antigen by the same procedure using an antibody immunoadsorbent column. The purity of the antigen isolated will depend on the purity of the original antiserum.

8.2 PURE CELLS

Cells can be isolated on the basis of any reactive molecules expressed on their surface membrane. These molecules can either be (a) specific receptors, for example antigen receptors, or (b) antigens, for example immunoglobulin molecules (which are also receptors), histocompatibility or blood-group antigens, etc.

In the first, and simplest, system we will describe cells are adsorbed to the immunoadsorbent on the basis of their membrane content of immunoglobulin, however these cells cannot be recovered and so we must work with the normal versus the depleted population. In the second system these cells may be recovered but in a slightly altered form; this is explained in Section 8.2.2.